A Double Layered Plate Method for the Detection of Microbial Lipolysis

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Summary. A double layered plating procedure has been adapted for the determination of lipolysis by micro-organisms. The base layer is prepared by mixing any fat, oil or triglyceride with Victoria Blue B and agar in a blender. Layers 2–3 mm thick are poured, allowed to solidify and overlaid with a suitable nutrient medium. This procedure allows considerable flexibility in the choice of fat as well as of nutrient medium. If lipolytic counts are desired, dilutions can be placed on the surface of the base layer prior to pouring the nutrient medium. Data are presented to show that no single fat or nutrient medium is suitable for all micro-organisms. Acid production from carbohydrates in the medium rarely caused confusion in recognizing lipolysis.

It is generally accepted that lipolysis occurs at the fat-water interface of an emulsion (Desnuelle & Savary, 1963); therefore, lipolytic activity increases in direct proportion to surface area until enzyme concentration becomes limiting. It is relatively easy to disperse a liquid fat into globules of 5μ or less with high speed blenders, but maintaining them in this state for the time necessary to make a series of dilution counts is often a problem. Emulsifiers can be used but some of them, e.g. bile salts, may be toxic; others, e.g. Tweens or lecithin, may serve as substrates, and natural gums, e.g., gum arabic, are tedious to prepare.

In addition to concern for size of globules, however, any procedure designed to enumerate or characterize lipolytic micro-organisms should have considerable flexibility in terms of lipid substrate, nutrient medium and numbers of samples or cultures to be studied. Many methods have been proposed and these have been reviewed by Rath (1961) and Muys & Willemse (1965). In most of them, one or more of the above problems is encountered. Fryer, Lawrence & Reiter (1967) recently employed a tissue impregnated with milk fat and Victoria Blue as a lower layer, as well as a tributyrin agar double layer procedure, in studying bacterial lipolysis. They found the double layer technique to be very useful and observed no inhibition by the Victoria Blue. However, no differentiation of type of Victoria Blue was made, and only milk fat and one kind of overlay medium were used with the Victoria Blue.

The procedure described here involves the use of a double layered plate. A base layer containing finely dispersed fat globules held in a non-nutritive agar matrix without the use of an emulsifier is overlaid with a nutrient medium. Differences in the lipolytic activities among microbial species with respect to nutrients, lipid substrate and incubation temperature are relatively easy to demonstrate with this method.

Materials and Methods

Removal of free fatty acids from substrate

The following triglycerides and fats were examined for their suitability as substrates: tributyrin, trihexanoin, trioctanoin, triolein, lard, butter oil, cotton-seed oil, olive oil and corn oil. It was observed early in the work that the colour of the background of agar plates, as well as of the lipolytic zones observed, varied with different lots of the same fat as well as with different fats. Varying amounts of free fatty acids were shown to be present in these triglycerides and fats. Since the indication of lipolysis is the production of a contrasting deep blue zone on a light blue background, constancy in colour of background and zone are desirable. Therefore, before use all lipids were dissolved in light petroleum (b.p. 30–60°) and passed through a column of activated alumina (chromatographic alumina, F-20, Alcoa, Bauxite, Arkansas, U.S.A.) to remove the free fatty acids.

Preparation of base layer

The base layer medium contained (%): fat, 5; 1:1500 Victoria Blue B solution, 20; agar (Difco), 1·5; water, 73·5. In one series of experiments, other types of Victoria Blue were used, and in another series, 0·85% of Oxoid Ionagar No. 2 was used. The above ingredients were sterilized separately, cooled to 50° and mixed in a warm, sterile blender (15,000 rev/min) for 1 min. Immediately after blending, 4–6 ml of the mixture were poured into Petri dishes, 100 mm diam, giving a layer 2–3 mm thick. With expeditious handling, the plates were solidified within 1–2 min after emulsification. With plastic Petri dishes, a larger volume was necessary because of the difficulty in wetting the surface with the fat-containing medium. Tubes of the individual, sterile ingredients may be stored in the refrigerator for at least 1–2 months prior to use.

Preparation of nutrient overlay

Although any agar medium suitable for growth and lipase production can be used, the following media were employed in this investigation: 1% peptone agar (Alford & Smith, 1965), Trypticase-soya agar BBL (Baltimore Biological Laboratories Inc., Baltimore, Maryland), or peptone agar containing 0·1% of yeast extract and varying concentrations of glucose. In some trials, Oxoid Ionagar No. 2 at a concentration of 0·85% was employed in place of 1·5% of Difco agar. Media usually were adjusted to pH 7, although in a few trials media adjusted to pH 6 or 8 were used. For streak plates a 2–3 mm layer of the nutrient overlay was poured over the solidified base layer. After hardening, these were inoculated with the desired cultures. If colonies were to be enumerated, the appropriate dilution of sample was placed on the solidified base layer and the melted overlay medium poured and mixed with the dilution. Counts also could be made by spreading appropriate dilutions of samples over the surface of the overlay medium.

Comparison of Victoria Blue preparations

Three chemically defined forms of Victoria Blue have been recognized for many years (Conn, 1946), but references to the use of this dye as an indicator of bacterial

lipolysis (Jones & Richards, 1952; Rath, 1961; Hugo & Beveridge, 1962; Muys & Willemse, 1965) refer to it simply as Victoria Blue. In this investigation, commercially available preparations of Victoria Blue, Victoria Blue B, Victoria Blue R, and Victoria Blue 4R (all manufactured by National Aniline, Co., New York) were examined for type of reaction given when included in media prepared as indicated below. Plates were overlaid with appropriate media and streaked with known lipolytic bacteria. Observations were made at 1, 2, 3, 5 and 7 day intervals for colour and width of zone around the colony, as well as background colour.

Effect of acid production

Since the Victoria Blue acts as an acid indicator, acid production by the micro-organisms from carbohydrates in the medium has been implicated as a possible cause of false positive reactions. To explore this point further, a peptone-yeast extract medium containing various concentrations of glucose was prepared and used as the nutrient overlay on lard, soyabean oil or tributyrin. These plates were streaked with several different strains of bacteria and on one plate of each medium was placed an absorbent disc saturated with 1% of either lactic acid or acetic acid.

Results

Selection of type of Victoria Blue

Emulsions of lard, tributyrin, soyabean oil and cacao butter were prepared with the 4 Victoria Blue preparations and streaked with several bacteria and moulds known to be lipolytic. These glycerides include a wide range of fatty acids in terms of chain length and degree of unsaturation of the fatty acids they contain. The data in Table 1 show the type of reactions to be expected with these indicators. Except for tributyrin,

TABLE 1

Colour reactions of different Victoria Blue preparations in agar media

Indicator dye	Colour obtained
Victoria Blue	Pale blue background; zones weak, but distinct
Victoria Blue B	Blue background; dark, sharp zones of lipolysis
Victoria Blue R	Pale blue; indistinct zones
Victoria Blue 4R	Mauve purple background, zones distinct but not as sharp as with Victoria Blue B

the type of glyceride had little effect on the reactions observed. Tributyrin usually showed a clearing of the medium rather than a blue zone, and the tributyrin background, with all indicators, was mauve coloured. From these comparisons Victoria Blue B, which displayed sharp, dark zones of lipolysis on a blue background, was selected as the dye of choice.

Variation in lipolytic response

Type of fat, nutrient substrate, and incubation temperature have been reported to be important in quantitative measurements of lipase production (Alford, Pierce & Suggs, 1964). To determine the effect of these factors in this qualitative plate method, 18 different media were prepared from the 9 triglycerides and fats, with an overlay of either Trypticase-soya agar or peptone agar. Plates of these media were inoculated in quadruplicate with 28 lipolytic micro-organisms including one or more lipolytic strains of *Pseudomonas fragi*, *Ps. fluorescens*, *Ps. perolans*, *Ps. ovalis*, *Ps. aeruginosa*, an unidentified *Pseudomonas* sp., a *Staphylococcus aureus*, *Geotrichum candidum*, *Penicillium roquefortii*, *Pen. camembertii*, *Mycotorula lipolytica*, *Rhizopus oligosporus*, *Thamnidium elegans*, *Endomyces vernalis*, and an *Aspergillus* sp. Two plates of each medium were incubated at 20° and two at 32°. Thus, an organism which was able to hydrolyse all substrates with both nutrient overlays and at both temperatures would give 36 positive reactions. The data in Table 2 show that only two micro-organisms had this versatility. In fact, only half of the cultures were positive in

TABLE 2

Variation in lipolytic responses among 28 micro-organisms

No. of positive reactions*	No. of cultures
36	2
31–35	8
26–30	3
21–25	3
16–20	5
11–15	3
5–10	3
0	1

* All cultures were streaked on 36 plates, representing 9 fats, 2 overlays and 2 incubation temperatures (see text).

>70% of the tests. Of more significance, however, is the recognition that had a single set of conditions been selected, it is possible that <50% of the cultures would have been recorded as lipolytic. In one experiment the initial pH of the nutrient overlays was adjusted to pH 6, 7 or 8; similar adjustments were made in the base layers. These were streaked with known lipolytic cultures and incubated at appropriate temperatures. Although there were slight differences in intensity and shade of both background and zone colours, these differences did not interfere with the detection of lipolysis.

Comparison of fat substrates and nutrient overlays

Forty four mould cultures isolated from cured and aged meats by Dr. L. Leistner at Iowa State University were examined for their lipolytic activity on various lipid substrates. The data in Table 3 indicate that more cultures were able to hydrolyse

tributyrin than were able to attack the fats containing predominantly long chain fatty acids. There were a few cultures, however, that attacked tributyrin poorly, if at all. In another experiment, lipolytic counts and total counts were made on 43 samples of fresh and cured meat products employing tributyrin, soyabean oil, lard or corn oil as lipid substrates and peptone or Trypticase-soya agars as nutrient overlays. No general preference for any one lipid was observed in an overall survey of the lipolytic count.

TABLE 3

Lipolytic activity of 44 mould cultures isolated from aged hams

Lipid substrate	No. of cultures showing lipolysis*
Tributyrin	32
Soyabean oil	22
Lard	15
Corn oil	22

* Four of the isolates were not lipolytic on any substrate.

TABLE 4

Effect of type of medium overlay on lipolytic and total counts

No. of samples showing					
Lipolytic counts			Total counts		
Higher on Trypticase-soya agar	Higher on peptone agar	No difference	Higher on Trypticase-soya agar	Higher on peptone agar	No difference
8	23	13	36	13	35

TABLE 5

Types of lipolysis produced in different agar media

Agent	Lipolysis produced					
	In peptone-yeast extract medium				In tributyrin agar	
	alone		+1% of glucose		alone	+1% of glucose
	A	B	A	B	C	C
Lactic acid (1%)†	+	−	+	−	−	−
Micrococcus freudenreichi	−	+	+	+	+	+
Cladosporium malorum	only slight growth		+	−	only slight growth	+
Geotrichum candidum	+	+	+	+	±‡	±
Cladosporium resinae	+	+	+	+	+	+
Lactobacillus casei	−	+	+	+	+	+
Streptococcus lactis	no growth		+	−	no growth	−
Pseudomonas fragi	−	+	−	+	+	+
Pediococcus cerevisiae	no growth		+	−§	no growth	−

A, diffused blue zone; B, distinct blue zone under or around streak when lard or soyabean oil was the fat substrate; C, clear zone.

†, Applied on an absorbent disc placed on the agar surface; ‡, narrow, clear zone, not evident until 3–4 days; §, when 1·5% of glucose was added, there was a distinct blue zone under the streak after 4–6 days.

The effect of type of medium overlay on lipolytic and total counts is shown in Table 4. In general, the higher total counts were obtained on the more nutritious Trypticase-soya agar while lipolytic counts were higher on the less nutritious peptone agar.

The data in Table 5 indicate that non-lipolytic carbohydrate fermenters would rarely give false positive tests. A blue zone is formed by water soluble acids, but this is rather diffuse and usually fades after a day or two. These zones should not be confused with the sharp zones caused by hydrolysis of triglycerides. The zones of lipolysis on tributyrin agar are clear, whereas zones of acidity from fermentation acids retain the blue colour.

No discernible differences in size or sharpness of zones were observed when Oxoid Ionagar was substituted for Difco agar.

Discussion

The data indicate that no single fat or nutrient overlay can be used for all micro-organisms, although tributyrin appears to be the most widely attacked of the triglycerides. However, some micro-organisms, such as *Geotrichum candidum*, produce a lipase which preferentially attacks lipid-containing unsaturated fatty acids and attacks lipid-containing saturated acids very slowly (Alford *et al.*, 1964). Thus, as was shown in Table 5, these organisms might be recorded as negative on tributyrin unless incubated for several days.

Muys & Willemse (1965) found that *Cladosporium suaveolens*, which requires carbohydrate for growth, would not grow on their Victoria Blue medium without carbohydrate, and concluded that a Victoria Blue medium would not be satisfactory for this kind of organism. However, they based their conclusion that a fermentable sugar was undesirable on an earlier observation of Jones & Richards (1952) with Night Blue. Rath (1961) observed very diffuse blue zones around non-lipolytic acid forming colonies grown on media containing fat, Victoria Blue and fermentable carbohydrate. He concluded, however, that the characteristic halo indicating lipolysis is distinct from the diffuse colour caused by acid formation from carbohydrates. The diffuse zones which we observed in this study when lactic or acetic acid or an acid producing culture was placed on the Victoria Blue media were similar.

Because the fermentation acids involved (acetic, lactic) are water soluble and gradually diffuse through the medium, the diffuse and gradually fading zone is to be expected. The higher fatty acids, however, are insoluble and remain essentially in the same place as the triglyceride from which they were released; this gives a sharp halo effect around the streak. Although tributyrin is insoluble, when it is hydrolysed the butyric acid which is released, being water soluble, might be expected to give a diffuse blue zone and thus make tributyrin an unsatisfactory substrate. However, since any monobutyrin formed is also soluble and dibutyrin is slightly soluble, a clear zone of hydrolysis surrounds the lipolytic culture. Why the blue colour of the Victoria Blue disappears is not entirely understood. However, since the dye is preferentially soluble in the glycerides as compared to water, it may be that when the insoluble triglyceride is converted to soluble mono- and diglycerides, the dye will diffuse along with them leaving a clear area.

In making a visual estimate of the effectiveness of an emulsification procedure, the interrelationships of volume, weight and surface area should be borne in mind. Although the surface area/unit wt increases as globule diameter decreases, it is sometimes overlooked that when the diameter of a globule is halved, it requires eight, not two, globules to contain the original weight of fat. Thus, an emulsion containing a few macroscopic globules may have only a small percentage of the total weight of fat in a finely dispersed state. When plates are prepared as described above, more than half the weight of the fat will be in globules of <5 μ diam.

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